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Attestation

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Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

98114201.1

091744641

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**Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation**

Anmeldung Nr.:
Application no.: **98114201.1**
Demande n°:

Anmeldetag:
Date of filing: **29/07/98**
Date de dépôt:

Anmelder:
Applicant(s):
Demandeur(s):
MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.
14195 Berlin
GERMANY

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
New cofactors for methyltransferases

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat:	Tag:	Aktenzeichen:
State:	Date:	File no.
Pays:	Date:	Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:

/

Am Anmeldetag benannte Vertragstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du dépôt:

Bemerkungen:
Remarks:
Remarques:

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Max-Planck-Gesellschaft zur Förderung
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Our Ref.: B 2286 EP



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29. Juli 1998

New cofactors for methyltransferases

The present invention refers to aziridine derivatives which can be used as cofactors for methyltransferases, complexes and compositions containing these compounds and their use for modifying a target molecule.

Nonradioactively labeled nucleic acids are of considerable interest in molecular biology, because they can be used in DNA sequencing and can serve as probes for Southern/Northern blots, *in situ* hybridizations and colony/plaque screenings without the potential health hazards of radioactive material. Several methods are presently known in the art of covalently modifying DNA and RNA (reviewed by C. Kessler in Nonisotopic DNA Probe Techniques, L. J. Kricka (Ed.), Academic Press, San Diego, 1992, pp. 29-92). For instance, modified oligonucleotides can be obtained by solid-phase DNA or RNA synthesis and the so modified oligodeoxynucleotides can be used as primers for a DNA polymerase (P. Richterich, G. M. Church, *Methods Enzym.* 1993, 218, 187-222). If the modification can not withstand the reaction conditions used in the solid-phase synthesis, incorporation of amine or thiol groups and postsynthetical labeling of the obtained oligonucleotides with amine or thiol reactive probes is possible (D. M. Jameson, W. H. Sawyer, *Methods Enzym.* 1995, 246, 283-300). In addition, several labels may be coupled to terminal phosphate or thiophosphate residues in oligonucleotides (J.-L. Mergny et al., *Nucleic Acids Res.* 1994, 22, 920-928).

Another method described in the art is the incorporation of modified deoxynucleosidetriphosphates into DNA with DNA polymerases (A. Waggoner, *Methods Enzym.* 1995, 246, 362-373) or with terminal deoxynucleotidyl transferase (L. K. Riley, M. E. Marshall, M. S. Coleman, *DNA* 1986, 5, 333-338; G. L. Trainor, M. A. Jensen, *Nucleic Acids Res.* 1988, 16, 11846).

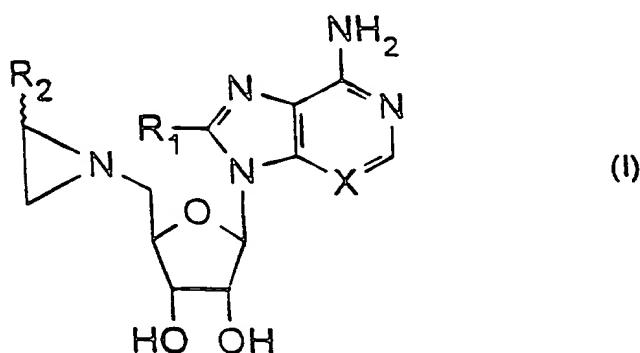
Furthermore, several modifications may be incorporated directly in DNA or RNA. For example, cytosine residues can be modified by activation with bisulfite followed by coupling with aliphatic amines (R. P. Viscidi, *Methods Enzym.* 1990, 184, 600-607; D. E. Draper, L. Gold, *Biochemistry* 1980, 19, 1774-1781). In addition, other chemical reagents for labeling DNA and RNA are commercially available (FastTag, Vector, Burlingame, CA; Mirus Label IT, Pan Vera Corporation, Madison, WI). These later methods, however, do not result in quantitative and sequence specific modifications and thus complex mixtures are obtained.

Nonradioactive labeling of proteins is straightforward, because their cysteine and lysine residues react readily with a large variety of labeling reagents (M. Brinkley, *Bioconjugate Chem.* 1992, 3, 2-13; R. P. Haugland, *Handbook of Fluorescent Probes and Research Chemicals* 1996, Molecular Probes Inc., Eugene, OR). However, generally proteins contain many lysine or cysteine residues and labeling often results in complex mixtures which are difficult to analyze. Thus, the specific modification of proteins is even more difficult than that of DNA and RNA. One strategy to obtain specifically labeled proteins is to engineer a protein with a single cysteine residue by means of a mutagenesis; subsequently, this cysteine residue is modified for example with a fluorescent group (G. Haran, E. Haas, B. K. Szpikowska, M. T. Mas, *Proc. Natl. Acad. Sci. USA* 1992, 89, 11764-11768). Furthermore, unnatural amino acids may be incorporated into proteins by *in vitro* translation (V. W. Cornish, D. Mendel, P. G. Schultz, *Angew. Chem.* 1995, 107, 677-690; *Angew. Chem. Int. Ed. Engl.* 1995, 34, 620-630). However, this method cannot easily be carried out and it results in only a small amount of modified protein.

Another possibility is the preparation of modified proteins by chemical peptide synthesis (T. W. Muir, S. B. H. Kent, *Current Opinion in Biotechnology* 1993, 4, 420-427); however, it is generally restricted to the preparation of relatively short protein chains.

It is the object of the present application to overcome the drawbacks of the known methods and to provide novel compounds which enable modification of biomolecules (for instance labeling) in a simple and effective way by the use of a methyltransferase.

This object is achieved by aziridine derivatives represented by formula (I)



wherein R¹ and R² independently from each other are H, ³H, -NH(CH₂)_nNHR' or -NH(C₂H₅O)_nC₂H₅NHR', R' is selected from fluorophores, affinity tags or cross-linking agents, n=3, 4 or 5 and X is N or CH.

Figure 1 shows the anion exchange chromatography of the enzyme reaction of the example after different incubation times.

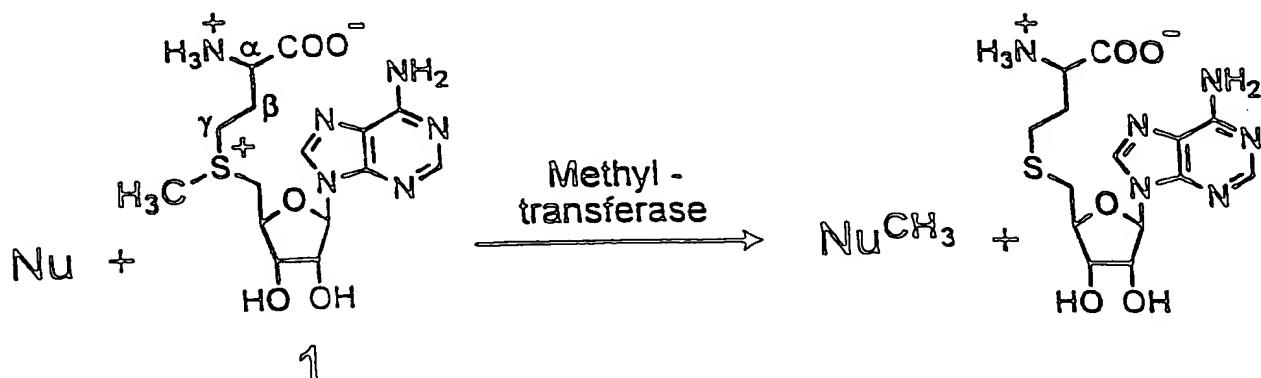
Figure 2 shows the electrospray mass spectrum of the product duplex oligodeoxynucleotide **4-5** eluted after 14.6 min.

The present invention will now be described in more detail.

S-Adenosyl-L-methionine-dependent methyltransferases (SAM-dependent methyltransferases) are a biologically important class of enzymes. They represent about 3% of the enzymes listed in the latest version of *Enzyme Nomenclature*, E. C. Webb, Academic Press, San Diego, 1992. They catalyze the transfer of the activated methyl group from the cofactor S-adenosyl-L-methionine to sulfur, nitrogen, oxygen and carbon nucleophiles of small molecules, phospholipids, proteins, RNA and DNA. For instance, DNA methyltransferases catalyze the methylation of the N6 position of adenine and the C5 or N4 position of cytosine within specific DNA sequences. Since restriction endonucleases are sensitive to DNA methylation, DNA methyltransferases can be used to decrease the number of restriction sites in DNA (M. Nelson, I. Schildkraut, *Methods Enzymol.* 1987, 155, 41-48).

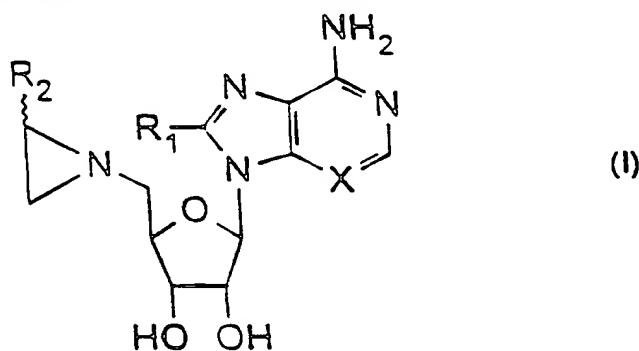
The reaction known to be catalyzed by SAM-dependent methyltransferases is shown schematically in the Reaction Scheme 1, where compound 1 is the cofactor S-adenosyl-L-methionine (SAM).

Reaction Scheme 1



The inventors of the present application have now found that the aziridine derivatives of Formula I below serve as cofactors for SAM-dependent methyltransferases and by this way enable the transfer of groups larger than methyl.

The aziridine derivatives of the present invention are represented by Formula (I)



wherein R¹ and R₂ independently from each other are H, ³H, NH(CH₂)_nNHR' or NH(C₂H₅O)_nC₂H₅NHR' and R' is selected from fluorophores, affinity tags or cross-linking agents, n=3, 4 or 5 and X is N or CH.

The term fluorophore as used herein is a chemical entity in which the electrons can be excited with light of a certain energy and photons with lower energy are emitted afterwards.

Preferred compounds of the present invention are those where R₁ and R₂ are each H or ³H and X is N.

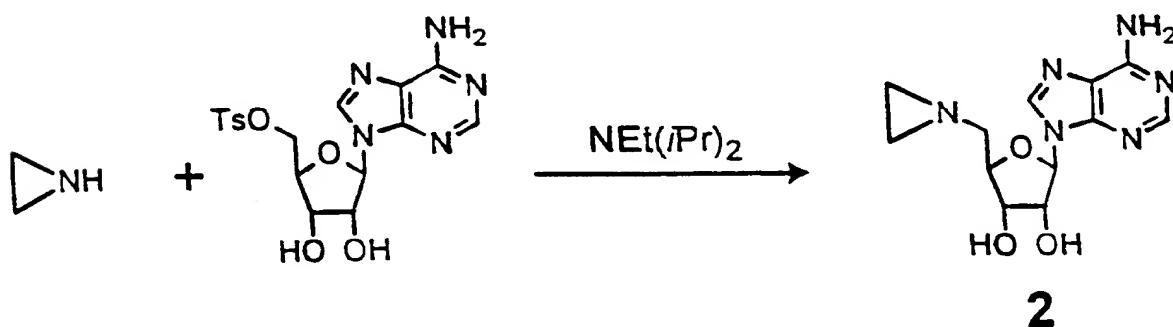
If R¹ and/or R² are NH(CH₂)_nNHR' or NH(C₂H₅O)_nC₂H₅NHR', R' is a fluorophore, an affinity tag or a crosslinking agent. Preferred fluorophores are BODIPY, coumarin, dansyl, fluorescein, mansyl, pyrene, rhodamine, Texas red, TNS and cyanine fluorophores like Cy2, Cy3, Cy3.5, Cy5, Cy5.5 and Cy7; derivatives of these fluorophores can also be used.

If R' is an affinity tag, it is preferably a peptide tag, biotin, digoxigenin or dinitrophenol; useful peptide tags are for example his-tag, strep-tag or glutathione.

Useful crosslinking agents are for example maleimide, iodacetamide, derivatives thereof, aldehyde derivatives and photocrosslinking agents. Examples for photocrosslinking agents are arylazide, diazo-compounds and benzophenone compounds.

N-Adenosylaziridine (compound 2) can for instance be synthesized in a one-step reaction by nucleophilic substitution of the tosylate group of 5'-deoxy-5'-tosyladenosine with aziridine (see Reaction Scheme 2 below).

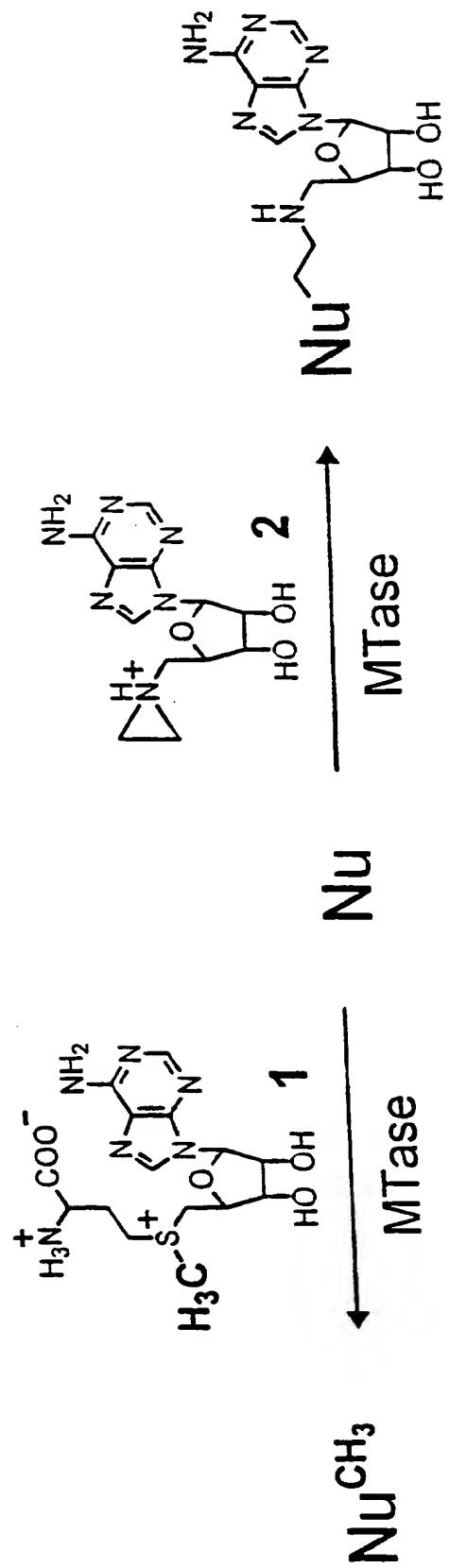
Reaction Scheme 2



Reaction Scheme 3 shows the reaction catalyzed by a methyltransferase (MTase) using the natural cofactor 1 and on the other hand using the new cofactor 2 according to the present invention.

6

Reaction Scheme 3

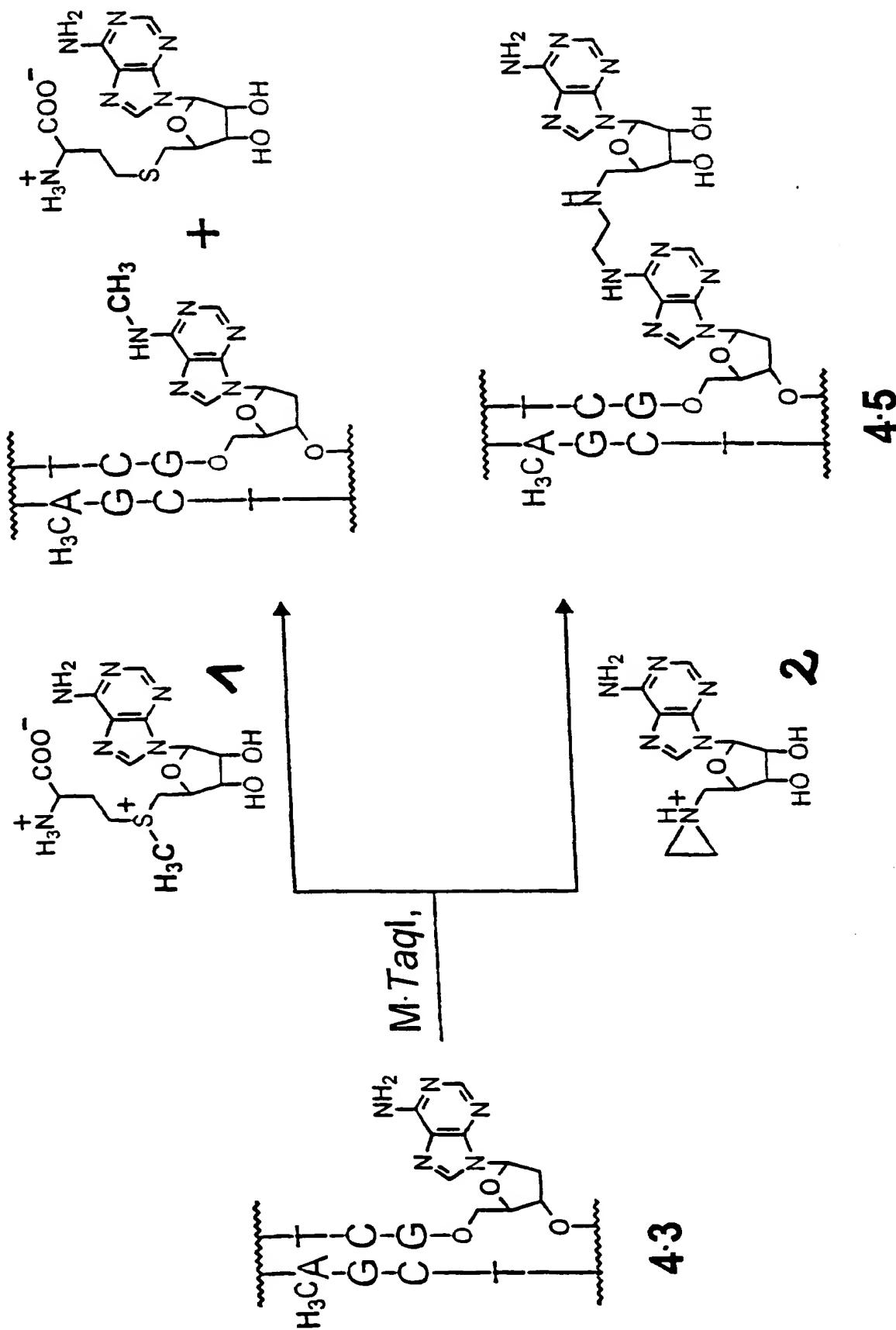


In Reaction Scheme 4, lower, the modification of a short duplex oligodeoxynucleotide (**4'3**), consisting of a plus strand oligodeoxynucleotide (5'-GCCGCTCGATGCCG-3', **3**) and a complementary minus strand oligodeoxynucleotide (5'-CGGCATCGA^{Me}GCGGC-3', **4**) with the protonated cofactor analogue **2** containing aziridine by the use of the adenine-specific DNA methyltransferase from *Thermus aquaticus* (M·TaqI) is shown. The complementary minus strand oligodeoxynucleotide **4** was chosen to contain N6-methyladenine-1-β-D-2'-deoxynucleoside (A^{Me}), which can not be further methylated by M·TaqI. M·TaqI usually catalyzes the methyl group transfer from the natural cofactor **1** to the exocyclic amino group of adenine within the double-stranded 5'-TCGA-3' DNA sequence (Scheme 4, upper) (M. McClelland, *Nucleic Acids Res.* 1981, 9, 6795-6804).

The structure of the reaction product **4'5** can for instance be verified by reversed phase HPLC-coupled electrospray ionization mass spectrometry (RP-HPLC/ESI-MS).

8

Reaction Scheme 4



Experimental results prove that with the unnatural cofactor **2** the non-methylated plus strand **3**, which contains an adenine at the target position within the 5'-TCGA-3' recognition sequence of M-TaqI, is modified quantitatively. Our observation that strand **4**, which contains N6-methyladenine at the other target position and an adenine outside the recognition sequence, is not modified, gives strong evidence that the base and sequence specificity of M-TaqI is not altered with the new cofactor **2**. In addition, this result provides indirect structural evidence that the target adenine of strand **3** is the site of modification.

This application for the first time describes the transfer of a group larger than a methyl group catalyzed by S-adenosyl-L-methionine-dependent methyltransferase. Since the transfer of for instance compound **2** introduces a unique secondary amino group into DNA, subsequent labeling reactions with amine reactive probes should be feasible. Thus, site-specific introduction of fluorescent, chemiluminescent or other reporter groups is possible. Alternatively, the new cofactors where R¹ and/or R² are NH(CH₂)_nNHR' or NH(C₂H₅O)_nC₂H₅NHR', with R' being a fluorophore, an affinity tag or a crosslinking agent, could be used to obtain site-specifically labeled DNA directly.

The present invention, however, is not restricted to M-TaqI but the C5-cytosine-specific DNA methyltransferase M.HhaI and other methyltransferases normally using S-adenosyl-L-methionine (SAM) as cofactor can also be used.

The methyltransferases useful in the present invention normally transfer the methyl group of SAM onto a nucleic acid molecule like DNA and RNA, onto a polypeptide or a small molecule. An overview on SAM-dependent methyltransferases is for instance given by R. M. Kagan and S. Clarke in *Archives of Biochemistry and Biophysics* 1994, 310, 417-427. This article also gives a list of small molecule O-methyltransferases and small molecule N-methyltransferases which include for example catechol O-methyltransferase and glycine N-methyltransferase.

Particularly preferred for use in the present invention are methyltransferases which are part of a restriction modification system of a bacterium.

The present invention not only refers to the aziridine derivatives themselves but also to the complex of such a derivative and a methyltransferase as well as pharmaceutical and diagnostic compositions comprising an aziridine derivative of the present invention or a complex thereof with a methyltransferase.

The aziridine derivatives of the present invention can be used for modifying a target molecule (e.g. DNA or fragments thereof, RNA or fragments thereof and polypeptides). This can be done by transferring an aziridine derivative of the present invention or a part thereof onto the target molecule by means of a methyltransferase.

The present invention will now be further illustrated by the following example.

Example

1. Synthesis of N-adenosylaziridine, compound 2

To a suspension of 5'-deoxy-5'-tosyladenosine (100 mg, 0.24 mmol, Aldrich) in N-ethyldiisopropylamine (125 µl, 0.7 mmol) under an argon atmosphere, dry aziridine (S. Gabriel, *Chem. Ber.* 1888, 21, 2664-2669; S. Gabriel, R. Stelzner, *Chem. Ber.* 1895, 28, 2929-2938) (360 µl, 7.2 mmol) was added slowly, and the resulting solution was stirred at room temperature for three days. Unreacted aziridine was removed under reduced pressure, and the crude reaction product was dissolved in water (1 ml), followed by neutralization with acetic acid (1 M). The solution (100 µl at a time) was injected on a reverse-phase HPLC column (ODS Hypersil 5 µm, 120 Å, 250 x 10 mm, Bischoff, Leonberg, Germany), and the product was eluted with a linear gradient of acetonitrile (7-10% in 30 min, 2 ml/min) in triethylammonium hydrogencarbonate buffer (0.1 M, pH 8.4). Fractions containing product (retention time 11.3 min, UV detection at 259 nm) were combined, concentrated by lyophilisation to 5.5 ml (10.5 mM, using $\lambda (\epsilon) = 260$ (15400) of adenosine) and stored at -80°C. Yield: 0.058 mmol (24%). For further characterization an aliquot was completely lyophilized to afford compound 2 as a white solid.

^1H NMR (500 MHz, D₂O): $\delta = 8.36$ (s, 1H; 2-H), 8.30 (s, 1H; 8-H), 6.13 (d, 1H, $^3J = 5.0$ Hz; 1'-H), 4.84 (dd = t, 1H, $^3J = 5.3$ Hz; 2'-H), 4.46 (dd = t, 1H, $^3J = 5.1$ Hz; 3'-H), 4.35 (ddd = dt, 1H, $^3J = 4.6, 4.6, 6.7$ Hz; 4'-H), 2.74 and 2.68 (AB part of ABX-spectrum, 2H, $^3J = 4.3, 6.6$ Hz, $^2J = 13.3$ Hz; 5'-Ha, 5'-Hb), 1.85-1.74 (m, 2H; aziridine H), 1.49-1.40 (m, 2H; aziridine H).

FAB-MS (thioglycolic acid): m/z (%): 293 (100) [$M^+ + H$], 250 (4) [$M^+ - C_2H_4N$], 178 (11) [$B^+ + C_2H_4O$], 167 (34), 165 (5), 164 (5) [$B^+ + CH_2O$], 158 (36) [$M^+ - B$], 149 (78), 136 (91) [BH_2^+], 102 (23); B = deprotonated adenine.

2. Synthesis and purification of oligodeoxynucleotides

Oligodeoxynucleotides **3** and **4** were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer, using standard β -cyanoethyl phosphoramidite chemistry. Syntheses were performed "trityl on" and oligodeoxynucleotides were purified by reversed phase HPLC. After detritylation with acetic acid (80%), **3** and **4** were further purified by reversed phase HPLC and desalting by gel filtration. The duplex oligodeoxynucleotide **4·3** was formed by incubating equal molar amounts of **3** and **4** in Tris acetate buffer (20 mM, pH 7.9) containing potassium acetate (50 mM) and magnesium acetate (10 mM) at 95°C (2 min) followed by slow cooling (2 h) to room temperature.

3. Enzyme reaction

The DNA methyltransferase M-TaqI free of cofactor was prepared as described by B. Holz, S. Klimasauskas, S. Serva, E. Weinhold, *Nucleic Acids Res.* 1998, 26, 1076-1083. The enzyme reaction was performed in a mixture (500 μ l) of M-TaqI 5 nmol; 10 μ M), duplex oligodeoxynucleotide **4·3** (5 nmol, 10 μ M), compound **2** (500 nmol, 1 mM) and Tris acetate (10 mM, pH 6.0), potassium acetate (50 mM), magnesium acetate (10 mM) and Triton X-100 (0.01%) at 37°C. The progress of the reaction was monitored by adding urea (100 μ l, 6 M) to aliquots (50 μ l) and injecting them on an anion exchange column (Poros. 10 HQ, 10 μ m, 4.6 x 100 mm, PerSeptive Biosystems, Wiesbaden, Germany). The compounds were eluted with potassium chloride (0.5 M for 5 min, followed by a linear gradient to 1 M in 30 min, 4 ml/min) in Tris hydrochloride buffer (10 mM, pH 7.6). The chromatogram of the anion exchange chromatography after different incubation times is shown in Figure 1.

RP-HPLC/ESI-MS: RP/HPLC/ESI-MS was performed using an ion trap mass spectrometer LCQ (Finnigan MAT, San Jose, USA) equipped with a high pressure gradient system (M480 and M300, Gynkotek, Germany). The product duplex oligodeoxynucleotide **4·5** was purified by anion exchange chromatography (see above) and desalting by repeated addition of water and

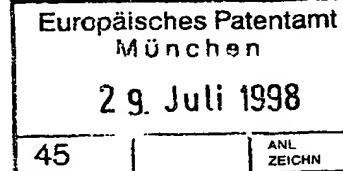
ultrafiltration (Microsep 3K, Pall Filtron, Northborough, MA, USA). Purified 4.5 was injected on a RP18 capillary column (Hypersil ODS, 3 µm, 150 x 0.3 mm, LC Packings, Amsterdam, Netherlands) and eluted with a linear gradient of acetonitrile (7-10% in 10 min, followed by 10-70% in 30 min, 150 µl/min) in triethylammonium acetate buffer (0.1 M, pH 7.0). Mass spectra were obtained using standard conditions.

The negative ion electrospray mass spectrum of the product oligodeoxynucleotide 4.5 eluted after 14.6 min is shown in Figure 2 and the chromatogram obtained by observing the total ion current is given in the inset. The molecular weights of oligodeoxynucleotides observed in the electrospray mass spectrum from Figure 2 are summarized in Table 1. In addition, the observed molecular weights of the educt oligodeoxynucleotides are given in Table 1.

Table 1

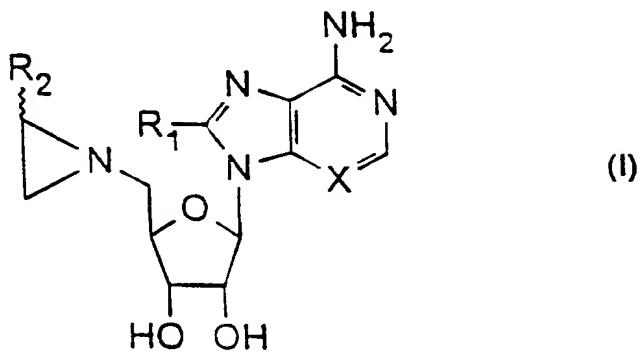
Oligodeoxy-nucleotide	Charge state	(<i>m/z</i>)exp	<i>M</i> exp	<i>M</i> theo
4.5	5-	1766.5	8837.5	8836.9
5	3-	1510.1	4533.3	4533.1
4	3-	1433.9	4304.7	4303.8
4.3	5-	1708.0	8545.0	8544.6
3	3-	1412.7	4241.1	4240.8

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Claims

1. Aziridine derivative represented by formula (I)



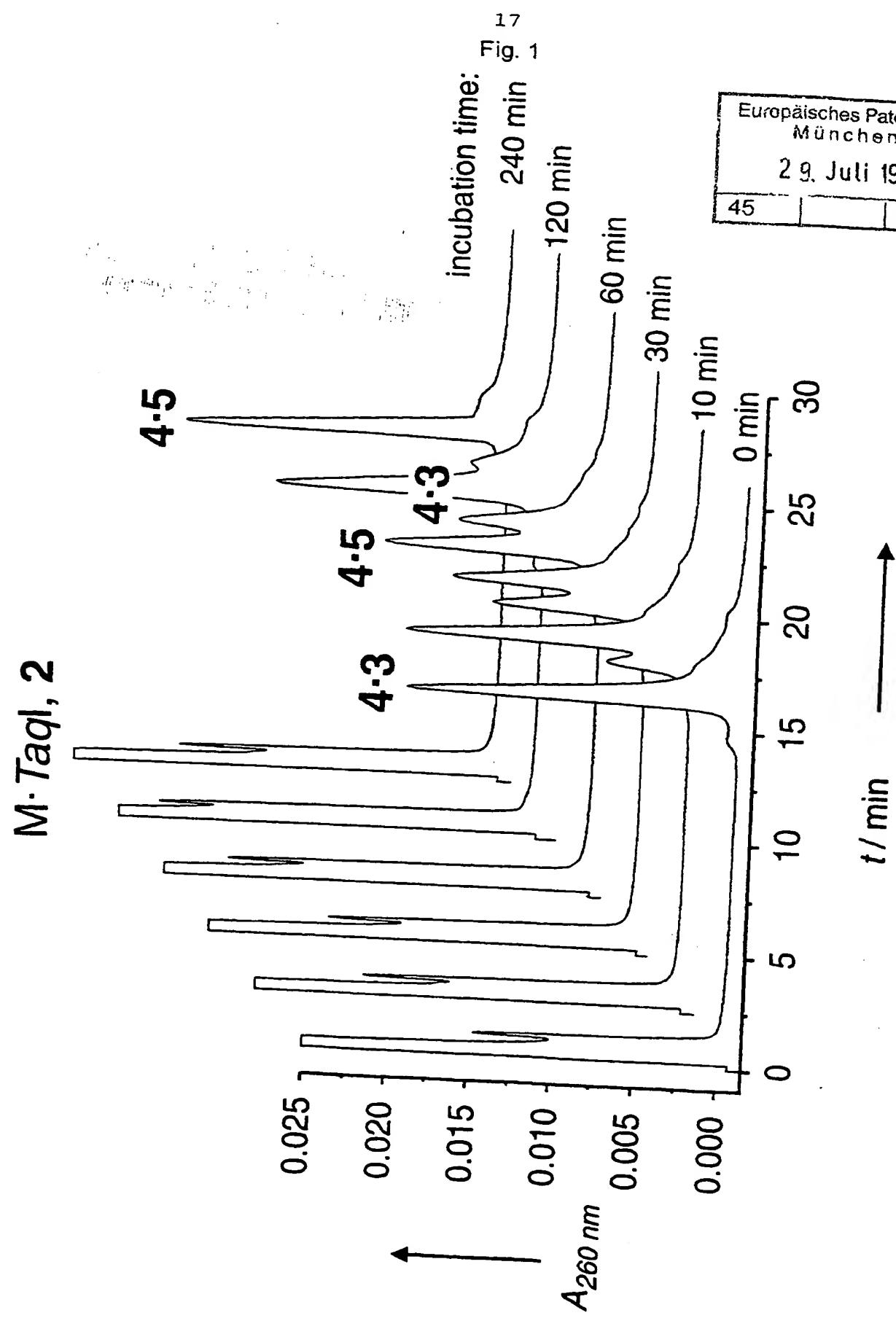
wherein R¹ and R² independently from each other are H, ³H, NH(CH₂)_nNH³H or NH(C₂H₅O)_nC₂H₅NHR' and R' is selected from fluorophores, affinity tags or cross-linking agents, n=3, 4 or 5 and X is N or CH.

2. Aziridine derivative of claim 1, wherein R₁ is H or ³H, R₂ is H or ³H and X is N.
3. Aziridine derivative of claim 1, wherein said fluorophore is selected from BODIPY, coumarin, dansyl, fluorescein, mansyl, pyrene, rhodamine, Texas red, TNS, the cyanine fluorophores Cy2, Cy3, Cy3.5, Cy5, Cy5.5 and Cy7, and derivatives thereof.
4. Aziridine derivative of claim 1, wherein said affinity tag is a peptide tag, biotin, digoxigenin or dinitrophenol.
5. Aziridine derivative of claim 4, wherein said peptide tag is his-tag, strep-tag or glutathione.
6. Aziridine derivative of claim 1, wherein said crosslinking agent is maleimide, iodacetamide, a derivative thereof or an aldehyde derivative, or a photocrosslinking agent.
7. Aziridine derivative of claim 6, wherein said photocrosslinking agent is an arylazide, a diazo compound or a benzophenone compound.

8. A complex of the compound of any one of claims 1 to 7 and a methyltransferase which normally uses S-adenoyl-L-methionine (SAM) as a cofactor.
9. The complex of claim 8, wherein said methyltransferase normally transfers the methyl group of SAM onto a nucleic acid molecule, a polypeptide or a small molecule.
10. The complex of claim 9, wherein said methyltransferase is part of a restriction modification system of a bacterium.
11. The complex of claim 10, wherein the methyltransferase is selected from the DNA methyltransferases M^rTaqI and M^rHhaI.
12. A kit comprising the compound of any one of claim 1 to 7.
13. The kit of claim 12 further comprising a methyltransferase as defined in any one of claims 8 to 11.
14. A kit comprising the complex of any one of claims 8 to 11.
15. A pharmaceutical composition comprising the compound of any one of claims 1 to 7 or the complex of any one of claims 8 to 11 and optionally a pharmaceutically acceptable carrier.
16. A diagnostic composition comprising the compound of any one of claims 1 to 7 or the complex of any one of claims 8 to 11.
17. Use of the compound of any one of claims 1 to 7 for modifying a target molecule.
18. The use of claim 17, wherein the modification of the target molecule is achieved by using the compound of any one of claims 1 to 7 as a cofactor of a methyltransferase which transfers the compound or part of the compound onto the target molecule.
19. The use of claim 17 or 18, wherein the target molecule is a nucleic acid molecule, a polypeptide or a small molecule.
20. The use of claim 19, wherein the nucleic acid molecule is DNA or RNA.

21. The use of claim 19, wherein the small molecule is a phospholipid.
22. The use of any one of claims 18 to 21 wherein the methyltransferase is a methyltransferase as defined in any one of claims 8 to 11.
23. A method for the preparation of a modified target molecule comprising the incubation of the target molecule with the compound of any one of claims 1 to 7 in the presence of a methyltransferase which is capable of using the compound as a cofactor and under conditions which allow the transfer of the compound or of part of it onto the target molecule.
24. The method of claim 23, wherein the methyltransferase is a methyltransferase as defined in any one of claims 8 to 11.
25. The method of claim 23 or 24, wherein the target molecule is as defined in any one of claims 19-21.

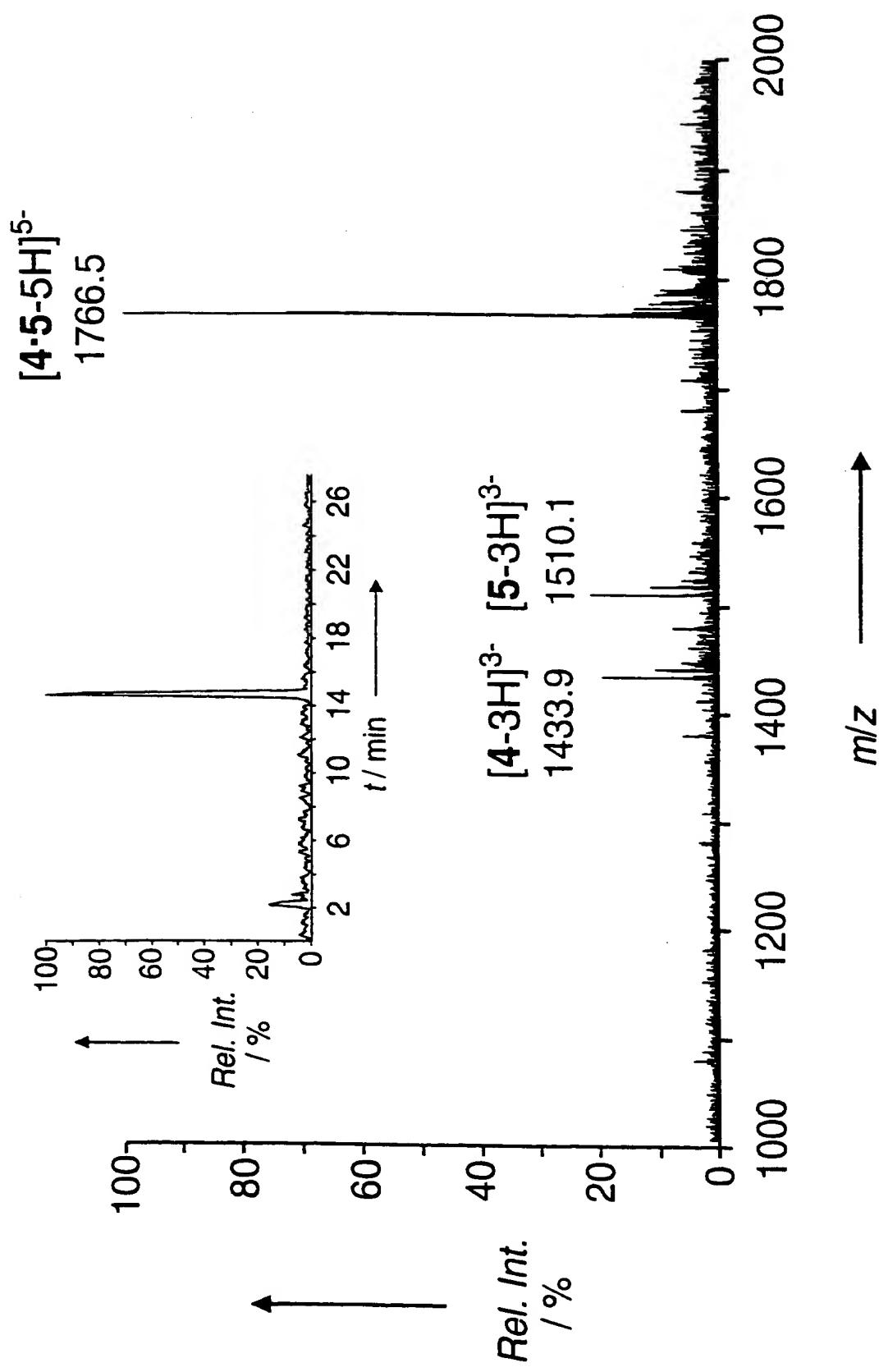
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DRAW

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Fig. 2

16

Abstract

New cofactors for methyltransferases

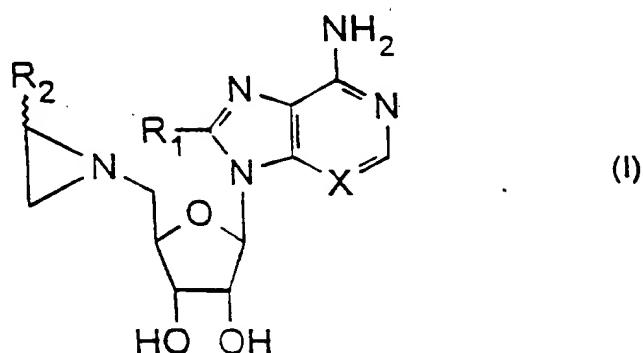
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29. Juli 1998

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Aziridine derivatives of formula (I)



are disclosed which can be used as cofactor for S-adenosyl-L-methionine-dependent methyltransferases.

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